Effect of repeated fever on growth in young guinea pigs

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science of Medicine (MSc Med).

Johannesburg, 2004

Dedication

This work is dedicated to my family

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science of Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Signature

6th day of May 2004

ACKNOWLEDGEMENTS

I am indebted to Professor Helen Laburn and Dr. Alida Faurie for their patient supervision and guidance throughout this study. The expert advise from Professor Duncan Mitchell and Dr. Andrea Fuller on statistical analysis was also highly appreciated. I also thank Mr David Makoa, and Mr Raymond Cherry for their technical assistance. The guidance of Professor John Pettifor of the Metabolic Unit of the Chris Hani - Baragwanath Hospital was also invaluable. Lastly, my husband Dr. Steve Madu deserves praise for *sponsoring me in this programme despite his other huge financial commitments and in all things may God take his glory for the life and hope that he has given me this day.*

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ABSTRACT

Repeated infection in early life can induce malnutrition and growth impairment due to the insufficiency of nutrients required to meet the increasing need for nutrients of a growing child, for growth. Infection causes an increase in metabolism and rate of tissue breakdown with a resultant need for extra nutrient intake. The aim of this study was to determine the effect of repeated fever on growth in young guinea pigs. Ten guinea pigs were studied from birth to approximately 60 days of age. At weaning age, guinea pigs were implanted with telemeters to measure body temperature. Then the guinea pigs were grouped into: An experimental group (n=5) receiving muramyl-dipeptide (MDP), and a control group (n=5) receiving normal saline injections. Eight injections per animal were given over the experimental period. Body weights of all animals were measured every 4 days while food intake was measured daily. Repeated fevers were observed in guinea pigs that received pyrogen injections. The rate of weight gain of the guinea pigs that received repeated pyrogen injections was significantly lower than the control group ($P < 0.05$). Food intake of the experimental group was also significantly lower ($P < 0.05$) on days of injections. However, there was no significant difference between the food-intake of the two groups on normal days. There was no difference in the blood concentration of albumin, glucose, IGF-1, iron, protein, and triglyceride, between the two groups. The serum zinc level of the pyrogen-injected group was significantly lower than the saline-injected group. Repeated fever during the growth phase of young guinea pigs resulted in reduced growth. Reduced food intake when fever was induced appear to be partly responsible for this reduced rate of growth.

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CHAPTER ONE

1. Literature Review

1.1 Fever and the 'Acute phase response' (APR)

Fever, an important and common symptom of infection can be defined as an elevation in core temperature that is above the normal range, as a result of a shift in the set-point, in response to pyrogen (Cooper 1995; Mitchell and Laburn 1985). Exogenous pyrogens such as bacteria and other toxins react with polymorphonuclear leukocytes, monocytes and certain reticuloendothelial cells to release endogenous pyrogen that is then transmitted to the hypothalamus (Cooper 1995), the major target site for the actions of the endogenous pyrogen (Hellon *et al*, 1991). The endogenous pyrogens such as interleukin-1 (IL-1), tumor necrosis factor (TNF) and interferons (Dinarello *et al.* 1984; Mitchell and Laburn 1985; Roth *et al.* 1997), act indirectly on the preoptic heat-sensitive neurons, increasing the set-point of the hypothalamic thermostat to febrile levels. Prostaglandins are thought to be the final mediators of fever and are associated with the increase of body temperature to febrile levels (Skarnes *et al.* 1981).

Infectious agents cause a host response known as the `acute phase response ` (APR) characterized not only by a rise in body temperature (Kluger 1991; Cooper 1995), but also a loss of appetite (Exton *et al.* 1995; McCarthy et al. 1984), weight-loss (Laugero *et al.* 2000; Moore et al. 1995; Beisel 1984), decrease in motor activities (Mitchell *et al.* 1997), initial fall in the number of neutrophils, lymphocytes, monocytes, and T-cells and

fall in blood concentrations of zinc, iron, protein and other nutrients (Cooper 1995; Beisel 1995; Langhans 1991) as well as a decrease in Insulin-like Growth Factor (Kaplanski *et al.* 2000; Beisel 1984; Cooper 1995).

1.2 Behavioural effects of fever: Infections are usually accompanied by behavioural changes collectively known as 'sickness behaviour' (Hart 1988). These include reduction in physical activity (Luker *et al.* 2000; Mitchell *et al.* 1997), depression, sleepiness, decrease in social interactions, hyperalgesia and decrease in food intake (Mitchell *et al.* 1997). Sickness behaviour results not only from natural infections but can also be induced experimentally by the injection of gram-negative lypopolysaccharide (LPS), cytokines such as IL-1β and gram-positive agents such as muramyl dipeptide MDP (Roth *et al*, 1997). During fever, IL-1β particularly has been found to mediate the inhibition of locomotor activity, grooming behaviour and reduction in food intake (Monkowski *et al.* 1997).

Anorexia: Anorexia or reduction in food intake is a common accompaniment to fever and a part of sickness behaviour (Langhans *et al.* 1991; Moore *et al.* 1995; McCarthy *et al.* 1984; Pekarek *et al.* 1971; Exton *et al.* 1995) and when food intake is reduced, metabolic responses such as the increase in proteolysis, glycogenolysis, and gluconeogenesis occur (Beisel *et al.* 1990). However, some evidence in experimental animals and humans suggests that the anorexia that accompanies fever may have some positive effects in recovery from infection (Langhans *et al.* 1993) or may have adverse effects such as malnutrition and the effect on immune functions and other defensive mechanisms (Exton *et al.* 1995; Langhans *et al.* 1993; McCarthy *et al.* 1984).

Decrease in motor activity: A reduction in activity level has been shown to occur after the injection of most cytokines especially IL-1β and TNF- α (Luker *et al.* 2000; Otterness *et al.* 1988; Bluthe *et al.* 1991). Decrease in activity may have a resultant effect of reducing food intake of the infected animals. However, the reduction in activity level could be a means for the host to minimize metabolic energy expenditure (Hart 1988).

1.3 Physiological Implication Of Fever:

During fever there is an increase in metabolism (Mitchell *et al.* 1990; Kluger 1986) and rate of tissue breakdown with resultant nitrogen loss creating a need for extra nutrients ((Mitchell *et al.* 1990; Beisel 1984). However, febrile subjects usually loose appetite (Langhans *et al.* 1991; Exton 1997) and food intake is grossly reduced during fever which may further increase the nutritional deficit in the growing child (Langhans *et al.* 1991; Davidson *et al.* 1975; Moore *et al.* 1995; Keplanski *et al.* 2000; McCarthy *et al.* 1984; Pekarek *et al.* 1975). Gram-positive bacteria or bacterial moities such as *Staphylococcus aureus* or Muramyl dipeptide have been successfully used in the recent times in the induction of fevers in experimental animals (Roth *et al.* 1997; Cooper 1995).

Muramyl dipeptide (MDP) is the minimal structure of peptidoglycans found mainly in the cell walls of Gram-positive bacteria (Roth *et al.* 1997). Muramyl dipeptide cause fever by direct action on the brain and by inducing endogenous pyrogen (Parant *et al.* 1980). The pyrogenicity of MDP is thought to be prostanoid mediated (Parent *et al.* 1980; Goelst 1991). Tolerance to LPS occurs particularly after daily injections (Kanoh *et al.* 1977) but can also occur when injections are made at few hours` or days` interval and normally

affects the second phase of fever (Atkins and Dinarello 1985). Repeated injection of MDP has been reliably shown not to cause tolerance in fever production in experimental animals (Roth *et al.* 1991).

Although the prevalence and effects of micronutrient deficiencies in growth retardation during infection have not been fully evaluated, alterations in the blood concentrations of zinc, glucose, protein, albumin and iron have been reported (Beisel 1977; Brown 1998; Golden *et al.* 1981; Hambidge *et al.* 1972; Michael *et al.* 1981; Mwangi *et al.* 1995; Ninh *et al.* 1996).

During the acute phase response, there are changes in the blood concentration of total protein, albumin, glucose and triglycerides (Beisel 1984; Cooper 1995) Catabolism of body proteins is the most visible example of the metabolic response to infection and is therefore an important characteristic of the acute phase response (Beisel 1984; Friman 1998). Various changes occur in the concentrations of plasma proteins during infection due to protein secretions or some pathological damage to the cells (Beisel 1977; Beisel 1984; Mousa *et al.* 1976). During fever, a negative nitrogen balance develops and continues throughout the active phase of the infection. The total nitrogen loss during the active phase of an infection is associated with the intensity and period of the fever (Beisel 1984). During infections, there is a change in the plasma levels of albumin. This may be due to increased rate of albumin degradation and redistribution to extra-vascular spaces or due to conditions of protein depletion and this occurs mostly in chronic infections (Mousa *et al.* 1976; Neufeld *et al.* 1978).

Glucose metabolism during infection is influenced by a variety of interacting factors and these include the effects of glucoregulatory hormones, availability of sugar from dietary sources, tissue stores or liver conversions of glycogen, and gluconeogenesis (Beisel *et al.* 1990). The onset of fever is followed by increased release of glucose from the liver. Shortly after fever onset, blood glucose rises to higher than normal concentrations and subsequently, slowly drops (Beisel 1977; Beisel *et al.* 1990). There is also impairment in glucose tolerance during fever, which causes an increase in the production of insulin to assist glucose uptake by the tissues (Beisel 1984; Friman 1998).

Infections of gram-negative organisms are usually accompanied by increase in the plasma levels of triglycerides. Most of the changes in the metabolism of lipids during infection have been shown to_occur in the liver (Sanchez *et al.* 2000; Beisel 1977) and the high blood level of triglycerides during infection may be due to excessive hepatic production and release, as well as difficulties in the triglyceride uptake (Keufmann *et al.* 1976).

Alteration in the blood concentrations of iron and zinc and total protein is a useful marker of the acute phase response as well as peripheral actions of endogenous pyrogens (Pekarek *et al.* 1975; Goelst and Laburn 1991). Iron is an essential element required for many of the cellular mechanisms involved in growth (Beisel 1981). Decrease in the serum concentration of iron has been consistently reported during the acute phase response (Beisel 1977; Cremades *et al.* 1985; Goelst *et al.* 1990; Kaplanski *et al.* 2000; Pekarek *et al.* 1975) partly due to release of an iron binder, lactoferin which is produced during neutrophil degranulation. The fall in plasma iron is thought to be beneficial to the host during infection because it may help to reduce bacterial replication by depriving them of an essential nutrient necessary for their growth (Hellon *et al.* 1991). During

infection, plasma iron enters storage sites such as the liver where it can be used in red blood cell production. A single episode of fever could cause a restriction in the release of iron from the red blood cell or cause a reduction in iron absorption from the intestinal tract resulting in a decrease in the serum iron concentration (Elin *et al.* 1977).

Zinc has been reported to support growth and development during early childhood and its deficiency is most clearly manifested in growth failure (Brown *et al.* 1998; Ninh *et al.* 1996; Sohlstrom *et al.* 1998). Both acute and chronic infections produce significant decrease in the plasma zinc concentration (Kaplanski *et al.* 2000; Pekarek *et al.* 1975).

Guinea pigs` growth is dependent on Insulin-like growth factor-1 (IGF-1) and results of experiments using animal models show similar changes in the IGF system observed in humans (Baumann 1997). IGFs are polypeptide growth factors produced in the liver and found in most tissues of the body are largely bound in circulation to binding proteins (IGFBPs). IGFs induce proliferation, differentiation and metabolic changes on a variety of cell types, and are essential for normal growth (Jones and Clemmons 1995; Van Wyk and Underwood 1978). IGF synthesis, secretion and blood concentration are regulated by nutritional and other factors (Thissen *et al.* 1999; Smith *et al.* 1995, Lang *et al.* 1997). During fasting, chronic food restriction or protein/energy malnutrition, IGF-1 concentration is reduced, but returns to normal within a few days after re-feeding (Smith et al, 1995; Thissen *et al.* 1999). Administration of endotoxin has been shown to cause changes in IGF-1 concentration in both humans and rodents (Abribat *et al.* 1993; Lang *et al.* 1997).

Effect of fever on growth: The catabolic nature of fever is reflected by weight loss and decrease in muscle mass and strength in the febrile subject (Laugero *et al.* 2000; Statakis *et al.* 1995; Moberg 1985; Moore *et al.* 1995). The rate at which any infection reduces growth is related to the severity and duration of the fever (Pereira *et al.* 1987; Eccles *et al.* 1989). Physiological and metabolic processes during infection/inflammation lead to losses in body nutrients that result in the reduction or cessation of growth in affected infants and children (Hauspie and Pagezy 1989; Cole *et al.* 1977; Pereira *et al.* 1987). These effects of fever on growth are in spite of the fact that there may be an increase in the endogenous production of anabolic hormones such as growth hormones (Beisel 1984) in the course of infection. The hyper-metabolic febrile state utilizes body constituents such as glycogen stores, fat deposits, and skeletal muscle proteins (Beisel 1984, 1990). Micronutrients and other blood substances such as iron, zinc, protein and glucose are thought to be important factors involved in the growth of the young (Davidson *et al.* 1975; Salgueiro *et al.* 2002). It is therefore useful to test for these substances during or after fever in a study of infection and growth. Growth impairment is a common manifestation in malnourished children as well as in children experiencing frequent infection in early childhood (Beisel 1984; Laugero *et al.* 2000; Eccles *et al.* 1989; Hall 2000). In infancy and early childhood, a single acute infection may have a decisive effect on the nutritional health and growth of the child, apparently because of the inability to meet the increased needs for protein and other nutrients necessary for growth (Beisel *et al.* 1977; Beisel 1984; Beisel *et al.* 1995; Laugero *et al.* 2000; Sohlstrom *et al.* 1998).

The etiology of growth impairment during infection is likely to be multi-factorial. In children plagued by a series of infections, growth impairment may be a consequence of

children plagued by a series of infections, growth impairment may be a consequence of increased metabolic requirement, altered food intake or changes in growth regulating hormones such as Insulin-like Growth Factor-1 (IGF-1) (Ketelslegers *et al.* 1995), (Sohlstrom *et al.* 1998).

1.4 Rationale:

Recruiting children into an experiment involving repeated injections of pyrogen would be difficult since parents may not be willing to give their children for such trials. Hence to mimic infection in children the animal model has been chosen for this study.

Guinea pig has been chosen for this study because many aspects of the febrile response are well described in this species (Blatteis 1977; Roth *et al.* 1997). The guinea pig is a small animal with a rapid growth and is therefore suitable for short-term experiments on growth.

Weight for age is an effective index used in assessment of growth and nutritional status especially in infancy when the measurement of length is difficult (Davidson *et al.* 1975). In this study therefore, body weight was monitored and used as an index for growth during the 30-day period of experiment.

1.5 Aims of the study:

The major aim of this study is to investigate the effects of repeated fever induced by the injections of the pyrogen Muramyl dipeptide (MDP), on growth of young guinea pigs using weight for age as the index of growth. In this study, I also intend to investigate the effect of repeated fever on food intake in young guinea pigs by measuring food consumed by the guinea pigs 24-hr before and 24-hr after the administration of the pyrogen.

At the end of the study blood samples were taken to investigate the effect of repeated fever on the blood concentrations of some relevant blood nutrients such as albumin, glucose, protein, iron, IGF-1, triglycerides and zinc.

CHAPTER TWO

2. METHODS

2.1 Animals and surgery.

Ten guinea pigs (Dunkin Hartley strain) from 3 different litters were used in this study. Birth weights ranged between 34 and 100 grams. The guinea pig pups were housed from birth in closed cages (18cm x 33cm x 55cm) with their mothers, in a room with a 12:12 hr light - dark cycle and an ambient dry bulb temperature of 22°C. The guinea pigs were fed a commercial diet of rabbit pellets *ad libitum* and allowed free access to tap water supplemented with vitamin C (500mg /l).

At approximately 23 days of age, surgery was performed on all guinea pig pups, and under general anaesthesia induced by intramuscular injections of 100mg/kg body weight Ketamine hydrochloride (Anaket-V injection, Centaur laboratories, South Africa) and Xylazine- 4mg/kg body weight (Chanazine 2% injection, Centaur laboratories, South Africa), temperature-sensitive radio-telemeters (Mini-Mitter, Sunriver, OR, USA) were implanted into their abdominal cavities.

2.2 Telemetry.

The radio- telemeters were prepared for implantation by waxing them with an inert wax (Elvax, MiniMitter, Sunriver, OR, USA) to make them waterproof, after which they were

calibrated in a water bath over a range of temperatures (35°C-40°C) against a precision quartz-crystal thermometer (Quat 100, Heraeus, Germany). Each waxed telemeter had a dimension of approximately 19 x 12mm and weighed approximately 3.0g each. The telemeters measured body temperatures of the animals to an accuracy of 0.1°C. The output frequencies from the telemeters were monitored by a receiver, RLA 3000 (Data Sci OR, USA) that was placed close to each guinea pig`s cage.

2.3 Experimental Procedure.

The guinea pig pups were allowed 7 days to recover from surgery before the start of experimental procedure. For the first 2 days post surgery, the pups were kept in the same cages with their mothers. Thereafter the pups were weaned from their mothers and placed into individual cages in a room with a 12:12hr light – dark cycle and an ambient dry bulb temperature of 22°C.

In guinea pigs, the rapid growth-phase occurs between birth and about 60 days of age. In a previous experiment, we monitored the growth of guinea pigs from birth until 60 days and showed a linear growth curve that flattened out from about 54days of age. We therefore decided to induce a series of fevers during this fast growth-phase, between day 30 and day 60 in the guinea pig.

The guinea pig pups were randomly divided into 2 groups: an experimental group (n=5) and a control group (n = 5) consisting of pups from three litters. The experimental group were given intra-muscular (im) injections of 50µg/kg of the pyrogen, muramyl dipeptide (MDP, Sigma) while the control group were given intra-muscular injections of 1ml of sterile saline (0.9% NaCl). Injections of MDP or saline started 5 days after the guinea pigs were weaned from their

mothers. At weaning, the guinea pig pups were about 26 days of age and on the day of the last injection they were 60 days of age.

Injections were administered at 9.00, on each day of injection. All guinea pigs were given a series of 8 injections spaced 4-5 days apart starting at about 30 days of age and ending at 60 days of age. On injection days, the guinea pigs were removed from their cages for weighing and injections and thereafter were returned and not disturbed for the rest of the day.

Body temperature:

On every day of injection, body temperature of all animals was measured every 15 minutes starting 1 hour before injections and continued until 6 hours after injections using a receiver that was gently placed close to the individual cage and out-put of the implanted telemeters were read off without disturbing the animals.

Body weight and food intake:

All guinea pigs were weighed every four days irrespective of whether the animals were getting injections or not. Weighing was done from birth until the end of the experiment, using an electronic mini-scale (Clover) that allowed the measurement of body weight to 1g accuracy.
Food intake was measured daily commencing 2 days before the start of experimental procedure. The guinea pigs were fed *ad libitum* and the remaining/wasted pellets after 24 hours, were manually separated from faeces by sorting. Then food intake over 24 hours was calculated.

2.4 Blood collection.

Eight days after the last injection of MDP or saline, guinea pigs were killed by an intra-peritoneal injection of sodium pentobarbitone, and blood was immediately sampled by cardiac puncture. The blood was centrifuged at 3000 r.p.m at 4^0C for 10 minutes. The serum obtained were frozen at -70°C until analysis. Serum concentrations of triglyceride, total protein, albumin, iron, zinc, IGF-1 and glucose, were analysed using methods shown in Table1.

Substance:	Method:
Albumin	Bromocresol green (BCG) Colorimetric assay with endpoint method (Doumas et al 1971)
Glucose	UV test using Glucose/HK kit (Tietz 1995)
Iron	Colorimetric assay based on Ferrozine method without proteinization. (Siedel et al 1984)
Total protein	Colorimetric assay using Biuret reagent. (Tietz 1995)
Triglyceride	Enzymatic Colorimetric test using triglyceride GPO-PAP kit. (Tietz 1995)
Zinc	Atomic absorption, flame (SANHLS)
IGF-1	Immunoenzymometric assay (IEMA) Kit (SANHLS)

Table 1. Methods of analysis of blood samples. * All analysis were done on serum samples. SANHLS = South African National Health Laboratory Service.

2.5 Data / Statistical analyses :

Changes in body temperature of animals after pyrogen or saline injections were used to calculate the fever index (FI). The FI was calculated as the difference between each 15-minute reading of abdominal temperature after injection (T_b), and the baseline abdominal temperature (T_o) (the mean body temperature measured over an hour before injection). The mean change in body temperature for each animal was multiplied by the total time over which abdominal temperature was monitored after injection (6 hrs). These 6-hourly fever indices were then represented by the equation FI = mean $(T_b - T_o)$ x 6, in °C.hr.

Rate of weight gain was calculated as the difference in body weight on each day of weighing compared to the weight at previous weighing day. Since body weights of all guinea pig were measured every 4 days, the rate of weight gain was expressed as weight gained over 4 days (g / 4 days).

Left-over food in the bowl, and food littered around the cages was collected and weighed. Thus total food intake over 24 hours was calculated.

All data are expressed as mean (±SD) and values of $P \leq 0.05$ were considered to be statistically significant. Two-way repeated-measures Analysis of Variance (ANOVA) was used to detect differences within each group (for instance, the series of calculated FIs in each group) and Student`s unpaired t-test was used to detect differences between two groups (for instance, food intake between the two groups).

2.6 Ethics:

The study was approved by the Animal Ethics Committee of the University of the Witwatersrand, South Africa (AESC number 98/99/4).

CHAPTER THREE

RESULTS

3.1 Body temperature responses

Figure 1 shows the typical abdominal temperature responses of two guinea pigs after an intra-muscular injection of either 1ml saline or 50µg/kg MDP

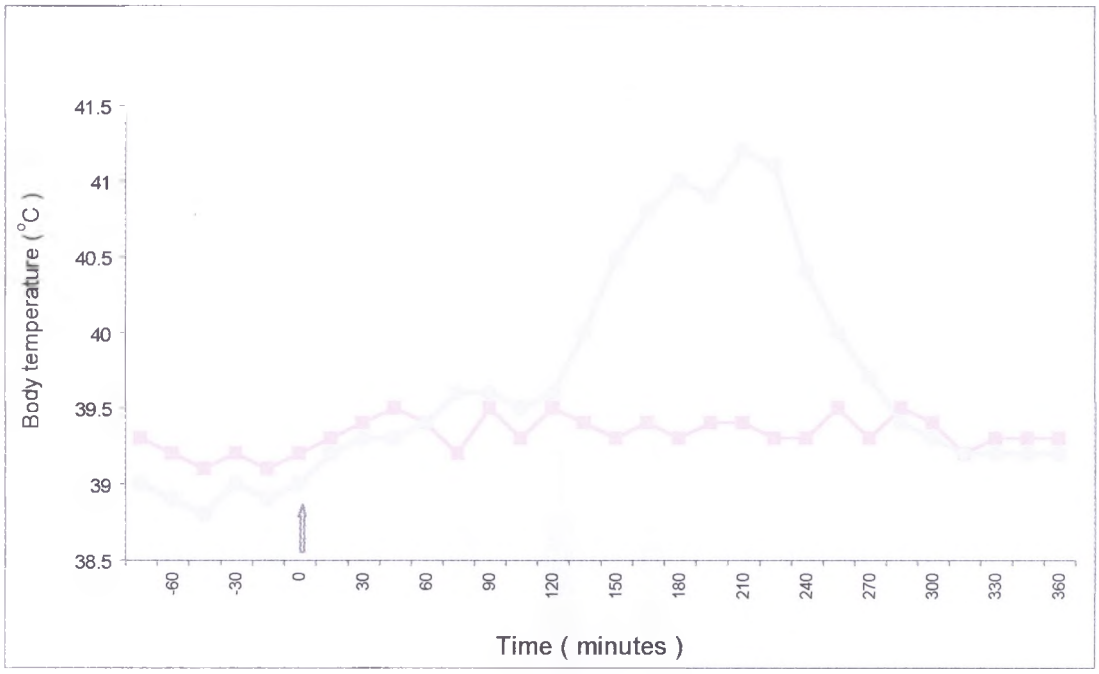

Figure 1. Typical body temperature changes in two individual guinea pigs following saline injection ( ) or MDP injection ( ). Ordinate, abdominal temperature in °C; Abscissa, time in minutes. ⇑ indicates time of injection.

Injection of MDP caused an increase in body temperature from about 120 minutes after the pyrogen injection, and peaked at increase of 1.6°C above pre-injection body temperature 195

minutes after injection. Some 60 minutes after reaching peak, temperature started to return to normal and were not different from that of the saline-injected by five hours after injection. There was no increase in body temperature of the guinea pig injected with saline.

Figure 2 shows the mean (± SD) Fever Index (FI) for each injection over the 30 days experimental period, in the two groups of guinea pigs. The overall mean FI of the MDP-injected guinea pigs was significantly higher (P = 0.0001, unpaired t-test) than that of the saline-injected group.

MDP causes FI of at least 3°C.hr on each occasion and there was no evidence of tolerance to the repeated injection of MDP when given at 4-5 days intervals (2-way ANOVA).

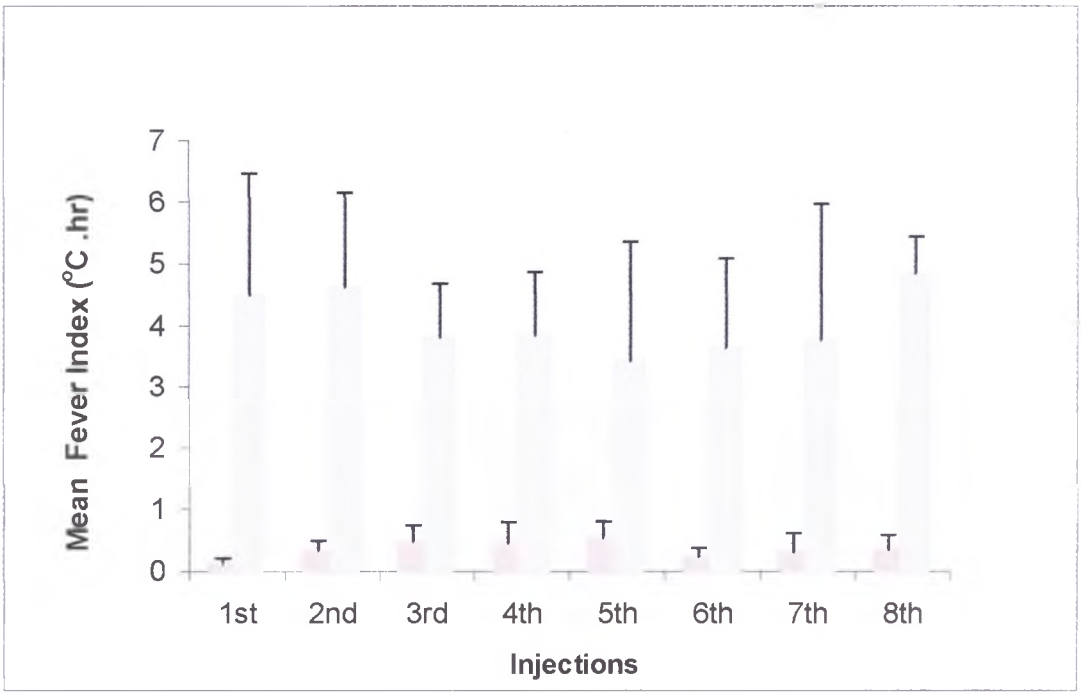

Figure 2. Mean (± SD) six hour fever indices calculated for each of the eight injections of either saline ( ) (n=5) or MDP ( ) (n=5). Ordinate, fever index in °C.hr; absissa, the series of injections, spaced 4-5 days apart.

There was no significant difference between the FI of the MDP group for each of the eight injections ($P<0.05$ Repeated Measures ANOVA with Bonferroni`s correction).

3.2 Effects on body weight.

Figure 3 shows mean changes in body weight of the two groups of guinea pigs from birth to 60 days of age. Birth weight for saline-injected animal was mean ±SD 50.6±18.8 and for MDP-injected animals was mean ±SD 88.2±10.5

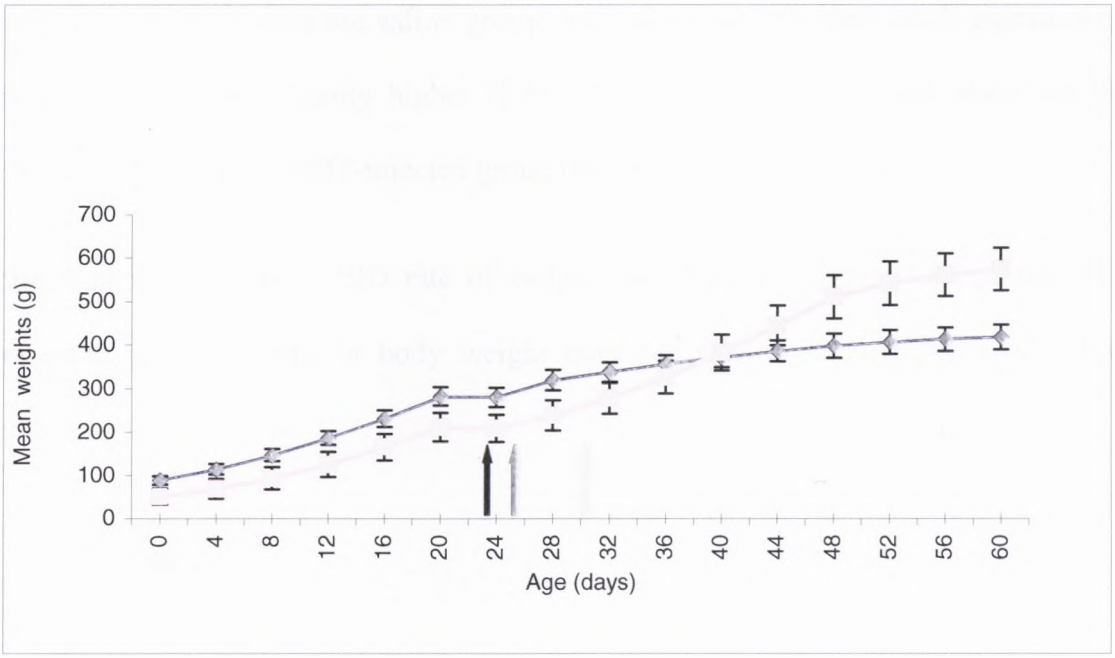

Figure 3. Mean (± SD) of body weights of the two groups of guinea pigs from birth until 60 days of age. , body weight of saline-injected group (n=5); , MDP group (n=5). Ordinate, body weight in g; abscissa, age in days from birth. ↑ indicate day of surgery; ↓ indicate day of weaning; points to the day of first injection.

All guinea pigs showed steady growth from birth until surgery. Disruption of growth was observed in all guinea pigs on days after surgery. However, the animals recovered from surgery after 5-7 days and this is evident in the continued increase in weight gain until the

start of injections at about thirty days of age. From the start of injections, a difference in the growth curve of the MDP and saline groups was seen. The MDP-injected group show a slower weight gain. The saline injected guinea pigs showed continuous increase in body weight until after the seventh injection at about 52 days of age when flattening was observed. Although the guinea pigs were randomly selected into two groups, it was observed that the guinea pig pups in the pyrogen group had significantly higher birth weights ($P = 0.0045$) than the saline group, and subsequent to birth of all guinea pigs, body weight was significantly higher ($P = 0.0079$) (unpaired t-test calculated on the slopes of the line) in the MDP-injected group than the saline, until surgery.

Figure 4 shows the mean ±SD rate of weight gain for the 2 groups of guinea pigs, represented as the change in body weight over the period of experiment at 4 days intervals.

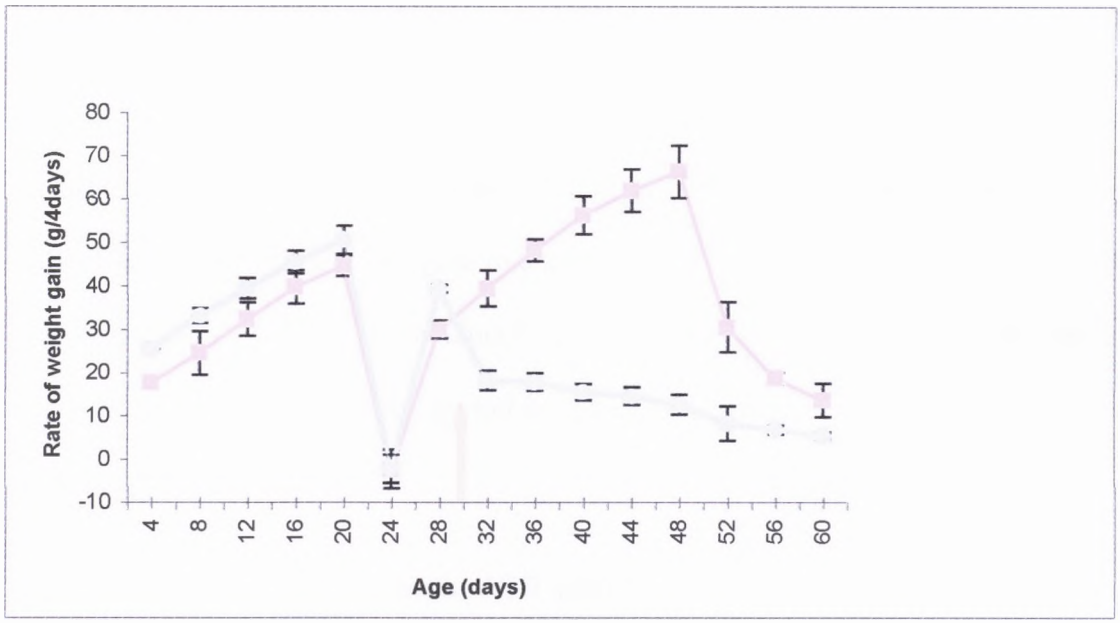

Figure 4. Mean ± SD rate of change in body weight calculated over at 4/day intervals from birth to 60 days of age. = saline-injected group (n=5); = MDP-injected group (n=5). Ordinate, rate of weight gain in g per 4 days; abscissa, age from birth in days. Surgery took place on day 23. points to the day of the start of injections.

Surgery caused a weight loss of about 1-3 g in all guinea pig pups. However, the pups recovered about 70% of their pre-surgery rate of body weight gain within 5-7 days of surgery. After the start of injections, the saline-injected group continued to gain weight at a rate significantly higher ($P < 0.05$, unpaired t-test) than the MDP group until about 52 days of age.

MDP-injected pups showed a decrease in the rate of weight gain of about 48-50% compared to the saline-injected, which caused a downward change in the slope of the

curve of the group. The decrease in the rate of weight gain of the MDP-injected guinea pigs caused the weight gain curves to separate progressively until the animals reached an adult-like rate of weight gain, from about day 50 of age.

The rate of weight gain of the saline group peaked at 45-48 days of age after which a decline in the rate of weight gain to about 35% was observed. However the rate of weight gain of the saline group remained significantly greater than that of the MDP group throughout the experimental period in spite of the decrease in the rate of weight gain of the saline-injected group.

Figure 5 shows that the mean ±SD total body weight gain over the period of eight injections was significantly lower ($P < 0.05$, unpaired t-test) in the pyrogen group than the saline group, with the saline-injected guinea pigs having a total weight gain of 315.8g, and the MDP-injected group a total weight gain of 99.2g over thirty days of experimental

period.

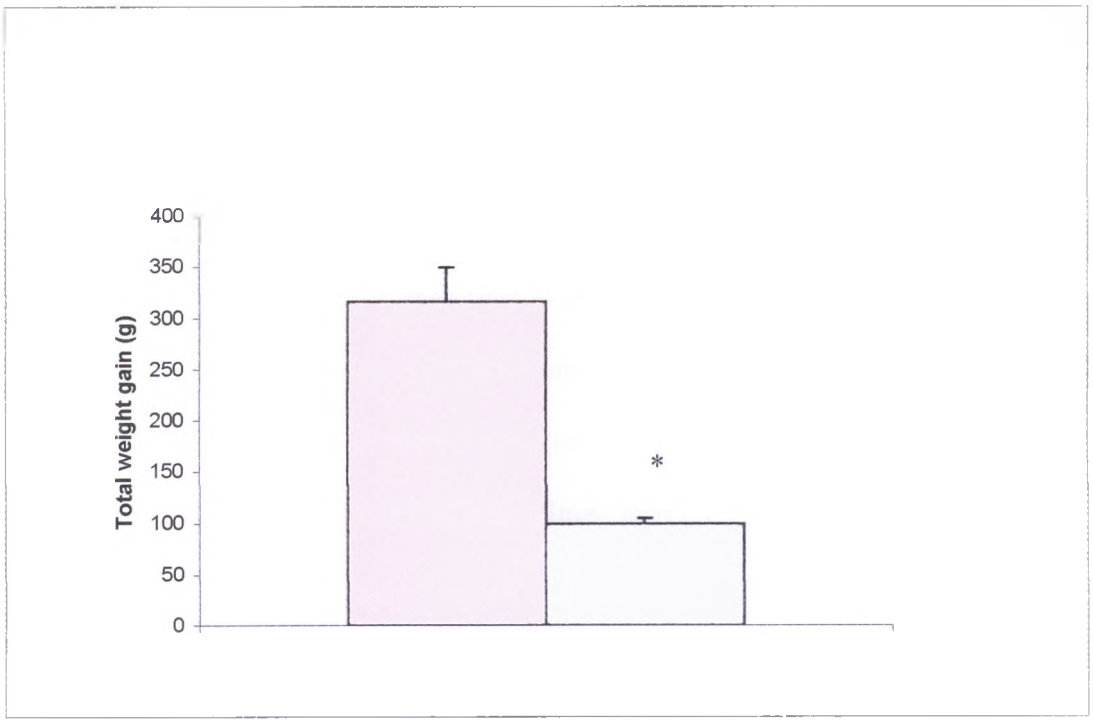

Figure 5. Mean ±SD total weight gain (from1st injection to last injection). = saline group (n=5). = MDP group (n=5). Ordinate, total weight gain over the experimental period in g. * show that saline group growth is higher than MDP group.

3.3 Food intake.

Figure 6 shows the mean ±SD of daily food intake for the 2 groups of guinea pigs from two days before the start of injections throughout the experimental period. Food intake in grams of the two groups of guinea pigs two days before the start of injections did not show any significant difference ($P < 0.05$, unpaired t-test). The food intake of the MDP-injected group showed a dramatic decrease of approximately 42% on each day of injection, while the food intake of the saline- injected guinea pigs did not show any decrease.

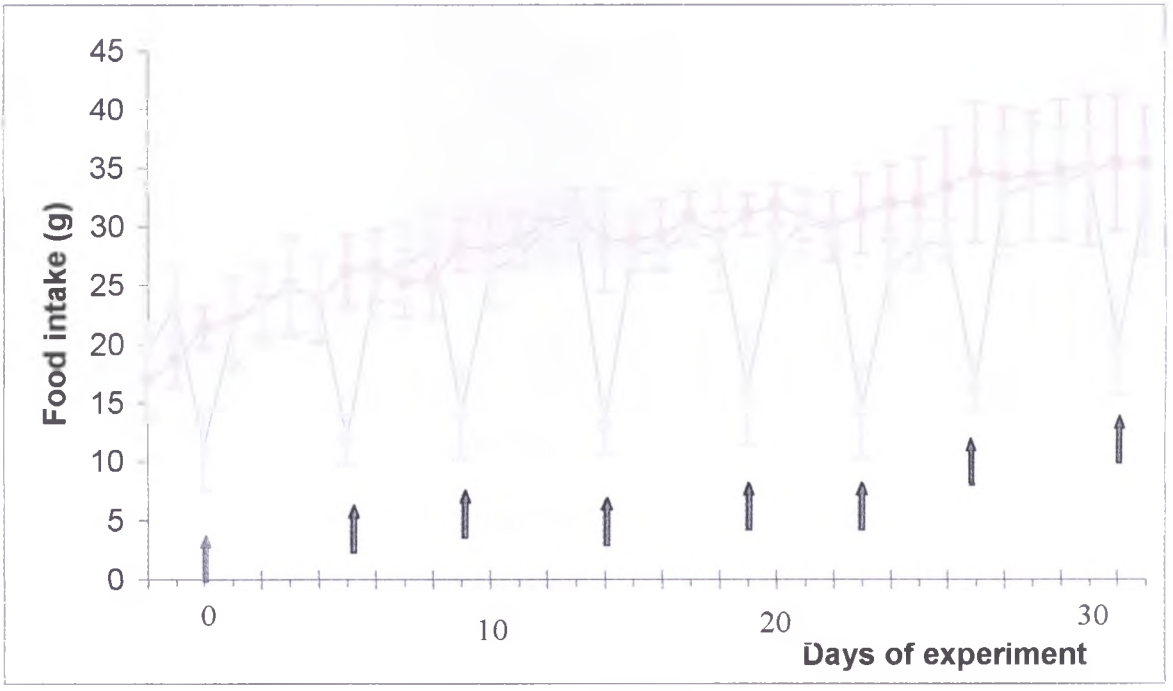

Figure 6. Mean ±SD of 24-hr food intake measured over a 30-day period in which eight injections of either saline (n=5,) or MDP (n=5,) were injected. Ordinate, food intake in g; abscissa, days of injection period. ⬆ = days of injection.

On days between injections, there was no significant difference (P = 0.5415, unpaired t-test) between the mean (±SD) food intake of the two groups.

The overall mean food intake over the experimental period was significantly higher (P = 0.0176, unpaired t-test) in the saline-injected group as shown in Figure 7. Intra-muscular injection of MDP caused a significant decrease in the food intake of the guinea pigs over the experimental period.

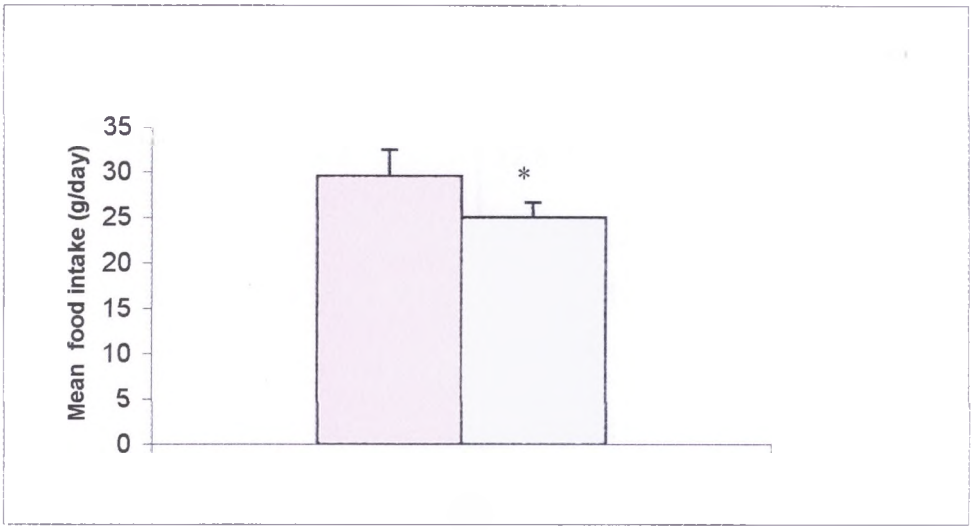

Figure 7. Mean ± SD total food intake from start of injection to end of injections. = total food intake of MDP group; = total food intake of saline group. * indicates values that are significantly different from the control.

3.4 Blood results.

Table 2 shows the mean ±SD values for zinc, albumin, glucose, IGF-1, iron, total protein and triglyceride from serum samples drawn eight days after the last injection of saline or MDP. No differences in the levels of albumin, glucose, IGF-1, protein, iron and triglyceride, were found between the two groups.

Test on serum	Saline (N=5)	Pyrogen (N=5)	P-value
Albumin (g/l)	23.6 ±1.5	23.0 ± 2.2	0.548
Glucose (mmol/l)	11.0± 5.2	12.8 ± 2.4	0.841
IGF-1 (µg/l)	105.9 ± 22.5	147.1 ± 17.0	0.179
Iron (mg/100ml)	208.2 ± 18.9	240.2 ± 41.6	0.157
Total protein (g/l)	42.0 ± 2.3	41.4 ± 4.0	0.691
Triglyceride (mmol/l)	0.3 ± 0.1	0.2 ± 0.0	0.299
Zinc (µmol/l)	20.8 ± 3.1	12.4 ± 2.6	0.0019 *

Table 1. Mean ± SD blood levels of Triglyceride, iron, zinc, total protein, albumin and glucose. denote value that are significantly different from the saline value.

However, a significantly higher ($P = 0.0019$, unpaired t-test) mean serum zinc concentration was found in blood taken at the end of the experiment from the saline-injected group

CHAPTER FOUR

DISCUSSION

This study has shown that intra-muscular (i.m) injections of MDP to young guinea pigs caused fevers with an average rise in body temperature of 1.5°C over about two and half hours. All eight successive fevers were comparable in size (Figure 3), signifying that repeated injections of MDP do not cause tolerance in young guinea pigs. Thus this pyrogen was an effective tool for the study of repeated simulated infectious episodes in guinea pigs.

The behaviour in the guinea pigs during fever appeared to also include reduction in activity levels, and a decrease in drinking. A decrease in the activity level, lack of social interaction and a host of other behavioural responses have been reported to occur as a result of natural infections, or after injection of pyrogens such as muramyl dipeptide (MDP) (Roth *et al.* 1997; Luker *et al.* 2000; Goelst *et al.* 1991) or cytokines such as Interleukine-1β (IL-1 β) and are collectively known as sickness behaviour (Mitchell and Laburn 1997; Hart 1988).

Although the amount of physical activity was not measured in this study, it was observed that after the injection of MDP, the guinea pigs had marked reduction in their physical movement in the cage as well as in feeding. Reduction in the physical activity during fever is thought to be due to the actions of cytokines such as IL-1β and tumour necrosis factor- α (TNF-α) (Luker *et al.* 2000). Night-time physical activity was significantly reduced during fever induced by the injection of *Staphylococcus*

aureus in rats showing that gram-positive bacterium (*Staphylococcus aureus*) similar to gram-negative bacteria (LPS), also produces fever and reduces physical activity at the time of the activity cycle when the activity level is high (Luker *et al.* 2000).

The reduction in the food intake of the pyrogen-injected guinea pigs occurred only on those days that the animals were injected with MDP and developed fevers. It is not known at what time after the injection of the pyrogen, the guinea pigs stopped feeding, seeing food intake was measured over a 24-hr period only. However, on days between injections food intake of the two groups were similar. The pyrogen-injected guinea pigs did therefore not show any `catch-up` in their food intake on the days between injections. As a result the total food intake of the pyrogen-injected group over the experimental period was significantly lower than that of the saline-injected group.

A decrease in food intake after administration of pyrogen suggests a role for cytokines in reducing feeding during fever (Langhans 2000). Many cytokines interact in a synergistic manner to induce anorexia during an infection. Interleukin-1-β is thought to induce activation of feeding-regulatory glucose responsive neurons in the ventromedial hypothalamus, a site involved in the integrative control of meal termination (Exton *et al.* 1995; Langhans *et al.* 1993). Anorexia caused by cytokines is also proposed to involve prostaglandins such as prostaglandin-E (Plata-Salaman 1996) and interferon-alpha (Langhans *et al.* 1993). The effects of cytokines on the central nervous system to cause anorexia, is thought to be through the actions and interactions of neurochemicals such as serotonin, histamine or dopamine (Langhans, 2000), and interleukin-6 (Luker *et al*, 2000), during infection. Suppression of feeding

as a part of sickness behaviour that occurs during infection can be beneficial or detrimental to the host depending on the time of the onset of the anorexia and the period for which it lasts during an infection (Exton *et al.* 1995; Plata-salaman 2001). Short term and temporary anorexia during acute infection may be beneficial to the host since reduction in the intake of nutrients such as iron, zinc, and protein, may stop bacterial growth (Exton *et al.* 1995; Exton 1997; Jones *et al.* 1977). However long term anorexia may lead to malnutrition and prolongation of disease (Plata-Salaman 1996).

In my study, the reduction in food intake on days of injections in the pyrogen-injected guinea pigs showed that as for the body temperature response, repeated injections of MDP did not cause a tolerance effect on anorexia. This result is in agreement with the work done by Langhans *et al.* (1990, 1991) in which they showed that repeated injections of MDP resulted in decrease in food intake, and also demonstrated no tolerance to reduced food intake of rats. Langhans and his colleagues also found that food intake on days between injections did not differ from the control. Similarly, decrease in food intake in lambs after repeated injections of yeast, has been demonstrated (Moore *et al.*1995), with an associated weight loss during the first two days after the injection. Similarly, Pereira *et al.* (1987) showed that fevers reduced food intake and caused weight loss in febrile children and furthermore showed that the duration of the anorexia was directly associated with degree of weight reduction in the children.

In my study, the greater rate of growth found in the MDP-injected guinea pigs before and after weaning could be associated with the greater birth weights of the group

compared to the saline-injected. Surgery not only caused a failure of growth, but weight loss of 1 to 3 grams in all guinea pigs. Although food intake was not measured at the time of surgery, food intake of the guinea pigs may have been reduced due to the effects of anaesthesia and most importantly, the surgery itself being a form of injury and stress. Surgery can initiate the activation of pro-inflammatory cytokines such as IL-1β whose actions may cause suppression of feeding and resultant weight loss observed after surgery. The rate of body weight gain of all animals returned to about three-quarter of their pre-surgery rate five to seven days after surgery.

The saline-injected group showed a steady and rapid growth rate from birth until 60 days of age. At about 48-50 days of age, they attained the highest rate of growth as measured over four-day period (Figure 5), after which there was a slowing down in their growth rate as measured by their rate of weight gain.

The mean rate of weight gain of the pyrogen-injected group followed a very different pattern after the start of injections (Figure 5). Having re-attained about two-third of the rate of weight gain after surgery, as in the control group, there was a dramatic reduction in the rate of growth from the start of MDP injections and the rate of weight gain remained significantly less than the rate in control animals for the period up to the last injection of MDP. The much flatter growth curve of the MDP-injected guinea pigs suggests that, with repeated fevers, guinea pigs do not demonstrate the ability for `catch-up` growth. The rate of growth after the start of the MDP injections was similar to the rate of growth of control guinea pigs of about 55-60 days` age, that is, the febrile guinea pigs apparently assumed a more adult-like growth rate. Our results are in agreement with Laugero *et al.* (2000) who showed that exposure to single

injection of LPS or behavioural stress can result in a decrease in growth in young mice, by reducing food intake and increasing basal energy expenditure. Contrary to the result of this study, Langhans *et al*, (1991) found that body weights of rats were not affected after repeated injections of MDP although they did not report on the rate of growth of the rats. The results of my study show however that the MDP-injected guinea pigs did not lose weight per se but had a decrease in their rate of growth compared to the control animals. The decrease in the rate of growth after repeated injections of MDP could have been due to a negative energy balance caused by the actions of IL-1 which through lipoprotein lipase inhibition, is thought to contribute to a negative nitrogen balance (Dinarello 988). Increase in energy expenditure, decrease in energy intake and alterations in the deposition of energy into lean and fat tissues could also be contributory factors to the suppression of growth seen in the MDP-injected guinea pigs (Laugero *et al.* 2000). It may have been possible for these guinea pigs to have an increase in their rate of weight gain in the days between injections but I did not measure daily rate of weight gain. Nevertheless, the repeated injections of MDP made recovery impossible and there was no apparent compensatory weight gain.

It is thought that infection causes an increase in circulating corticosterone which results in fat and protein catabolism thereby inhibiting growth and feed efficiency (Laugero *et al.* 2000). The increase in metabolic rate and increased energy expenditure by the animals to regulate body temperature during fever could (Mitchell and Laburn 1997) have resulted in a negative energy balance that causes the body to utilize body energy stores. My results agree with those of Hauspie and Pagezy (1989) and Cole *et al.* (1977) who found that children who get frequent or prolonged

infection during growth phase, may have growth impairment.

During fever, the decrease in the concentration of circulating Insulin-like Growth Factor-1 (IGF-1) (Ma *et al.* 2001; Ninh *et al.* 1996), may also be associated with decreased growth in both young children and experimental animals. Anorexia that occur during fever is as well known to affect growth especially in prolonged infection in children (Pereira *et al.* 1987; Eccles *et al.* 1989).

Malnutrition and infection are interrelated (Miall *et al.* 1970) as infection not only decreases nutrient intake, but alters nutrient metabolism (Pereira *et al.* 1987). Infection therefore becomes more serious in cases where the nutritional status of a child is poor (Miall *et al.* 1970). Good health in children is manifested in normal growth and repeatedly infected children usually grow at slower rates (Hall 2000). However, it has been reported that children have the tendency to grow more rapidly during recovering or after infections (Black 1991; Rowland *et al.* 1988; Briend *et al.* 1989). In contrast, other researchers have shown that there is no `catch-up` growth after infections (Wingen *et al.* 1999). Because of the energy demands during fever, it may be useful to keep febrile subjects warm during fever in cold environments so as to reduce the energy utilized by the body in generating and maintaining higher body temperature during fever. In cold climates, more energy is needed to raise body temperature while in hot climates increase in body temperature is achieved by increasing heat conservation thereby saving energy (Mitchell and Laburn 1997).

Consequently, it may therefore be important to treat fever in children to decrease the metabolic demand associated with maintaining an elevated body temperature. The use of antipyretics has the benefit of eliminating more than one aspect of the febrile

responses (Mitchell and Laburn 1997).

The similarities in the blood substances such as iron, glucose, triglyceride and albumin in the MDP-injected group and the control could be attributed to the time interval of 8 days between the last injection and blood sampling by cardiac puncture. The animals may have fully recovered from the effects of the pyrogen injections within the eight-day period after the last injection, and blood levels of these nutrients could have returned to normal. The similarities in the serum levels of albumin, triglyceride, total protein and glucose and iron in the pyrogen and saline-injected guinea pigs, is not in agreement with previous work (Cremades *et al.*, 1986; Elin *et al.* 1977; Goelst *et al.* 1991; Duggan *et al.* 2001; Lees *et al.* 1972; Exton et al. 1995), which have shown that fevers result in alterations in the blood concentrations of these substances. Infection is associated with a decrease in blood protein, which usually occurs early in the infection. At recovery stages and re-feeding, the protein levels return to normal. Sanchez *et al.* (2000) also demonstrated that alterations in the triglycerides levels occur during fever, depending on the duration of the fever.

The significant decrease in serum concentration of zinc in the pyrogen-injected guinea pigs could be attributed to increased diversion of zinc to the liver, that is associated with repeated pyrogen injections or infections (Brown 1998; Pekarek and Evans 1975). My results indicate that the effects of repeated pyrogenic injection on the zinc metabolism, are much more prolonged than effects on other substances. Zinc is an essential mineral found in almost every cell in the body and has been reported to support growth and development during childhood, and the results of zinc deficiency mainly manifest in growth failure. Many researchers have reported that inflammatory

stress and acute and chronic infections, result in significant changes in zinc metabolism in humans and experimental animals (Beisel *et al.* 1971, 1990, 1995; Pekarek *et a.l* 1975). Both acute and chronic infection produce significant increases in zinc retention by the liver, during the first 24 hours of stress (Pekarek and Beisel 1971; Pakerek and Evans 1975) with a resultant decrease in the serum concentration of zinc in infected animals. Decrease in the concentration of serum zinc can also be attributed to reduced nutrient intake that follows anorexia during infection (Kaplanski *et al.* 2000).

Several authors have shown that reduced serum zinc concentration is associated with a decrease in the rate of weight gain and experimental zinc deficiency is also associated with low blood levels of IGF-1 (Thissen *et al.* 1999). Hence the similarities in the concentration of IGF-1 levels between saline and MDP-injected animals did not meet expectations considering the well established association between zinc and IGF-1 levels during and after infection. However, the result could be explained in terms of the actions of the growth factor in catch-up growth that usually take place during recovery from infections. Although growth rate was not monitored after the end of injections, there is the possibility that the MDP-injected guinea pigs could have increased their rate of growth in compensation for the reduced rate of growth in the previous weeks. The eight-day recovery period might have allowed for the IGF-1 levels to normalise. In association to zinc, the result of my study could indicate a longer time frame for zinc to normalise than would the IGF-1 during recovery or a possible dissociation of the actions of zinc and IGF-1 in short term recovery.

The decrease in the serum zinc concentration and no decrease in serum iron, suggests that the zinc changes are not just a result of prolonged acute phase response (Mitchell and Laburn 1997) but were probably due to nutritional factors. It is possible that repeated fevers caused changes in zinc metabolism and not in other minerals or the decrease in food intake and a resulting decrease in weight gain were responsible for the reduced serum zinc concentration.

Interpretation of blood variables in my study is confounded by the long period of time that elapsed between the last fever induction and blood sampling as blood sampling by cardiac puncture could only occur at the end of the experimental period. Sampling blood 12-24 hours after each pyrogen/saline injection could be a more accurate way of determining the effect of repeated fevers on the blood concentrations of the micronutrients and other blood substances. IGF-binding proteins should also be measured in future related studies.

Conclusion.

The results of this study have shown that repeated intramuscular injections of the gram-positive pyrogen, MDP, to young guinea pigs results in successive fevers, dramatic reduction in food intake on days of febrile episodes, overall slower rate of weight gain and lower serum zinc concentration. While some authors argue that fever- induced anorexia is one of the important behavioural strategies that organisms have evolved for the fight against pathogenic invasion, it must not be overlooked that frequent infections have detrimental effects on the host by producing growth retardation at least in the young animal during its period of rapid growth. Further studies are required however, to determine the mechanisms by which simulated infection and fever reduce food intake and what proportion of reduced growth can be attributed to the anorexia itself, or other factors, such as the requirement for increased metabolic rate during fever generation. Moreover, the cause of a decrease in serum zinc concentration and the effect of a reduction in serum zinc concentration need to be elucidated. I would recommend that studies be carried out to establish whether zinc supplementation is of use in reducing the growth deficits experienced by the young suffering repeated infections.

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